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Accommodation of Pyrimidine Dimers During Replication of UV-Damaged Simian Virus 40 DNA

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UV irradiation of simian virus 40-infected cells at fluences between 20 and 60 J/m^2, which yield one to three pyrimidine dimers per simian virus 40 genome, leads to a fluence-dependent progressive decrease in simian virus 40 DNA replication as assayed by incorporation of [^{3}H]deoxyribosylthymine into viral DNA. We used a variety of biochemical and biophysical techniques to show that this decrease is due to a block in the progression of replicative-intermediate molecules to completed form I molecules, with a concomitant decrease in the entry of molecules into the replicating pool. Despite this UV-induced inhibition of replication, some pyrimidine dimer-containing molecules become fully replicated after UV irradiation. The fraction of completed molecules containing dimers goes up with time such that by 3 h after a UV fluence of 40 J/m^2, more than 50% of completed molecules contain pyrimidine dimers. We postulate that the cellular replication machinery can accommodate limited amounts of UV-induced damage and that the progressive decrease in simian virus 40 DNA synthesis after UV irradiation is due to the accumulation in the replication pool of blocked molecules containing levels of damage greater than that which can be tolerated.

We are studying replication of UV-damaged DNA in mammalian cells to obtain a basis for understanding molecular mechanisms of mutation induction. Mutations appear to occur when UV photoproducts remain in the DNA and interfere with normal DNA replication (7, 14). Previous work in other laboratories has shown that when mammalian cells are irradiated with UV radiation, DNA replication is inhibited and DNA strands synthesized after irradiation appear to be shorter than normal (reviewed in references 7 and 13). The size of the newly synthesized DNA strands correlates with the average distance between dimers in the template strands. To explain these results, it has been postulated that in mammalian cells, as in bacteria, pyrimidine dimers in the template strand block replication fork progression, but resumption of synthesis may occur beyond the dimer sites. However, further analysis of the molecular details of the replication of UV-damaged mammalian DNA has been quite difficult, due to the size and complexity of the mammalian genome and the fact that replication occurs simultaneously on many nonidentical replicons.

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To probe the molecular mechanism of replication of UV-damaged DNA in mammalian cells, we have chosen to study the simple, well-defined replicon of simian virus 40 (SV40). The SV40 replicon has been used extensively as a model for understanding normal cellular replication processes (3). Replication of SV40 DNA can be described in terms of the passage of molecules through three main molecular pools which exist simultaneously in the nucleus. Molecules that have initiated replication enter the pool of replicative intermediates. The replication process takes about 15 min (2), and two daughter molecules are produced which can either reenter the replication pool immediately or remain in the pool of completed form I molecules to be replicated later or packaged into virions (24). Once the DNA enters the pool of completed virions, it appears to be removed from further participation in replication.

We report here our examination of the replication of UV-damaged SV40 DNA. In our analysis, we have focused on the kinetics of production and characterization of newly replicated molecules after UV irradiation of infected cells to determine the extent to which UV damage interferes with normal DNA replication and whether some DNA damage can be tolerated by the replication machinery of the cell. Our experimental conditions were chosen to interfere as

little as possible with the replication machinery of the cell (i.e., we have avoided the use of temperature-sensitive mutants and temperature shifts). We used density labeling to allow distinction between molecules that become fully replicated after UV irradiation and those that were replicated or initiated before irradiation. This allows us to avoid uncertainties as to whether dimers found in newly replicated molecules are actually introduced behind the replication fork. Also, we used only low UV fluences such that the SV40 genomes will contain an average of only one to three pyrimidine dimers. Our conditions allow the examination of events occurring both immediately after UV irradiation and during subsequent rounds of replication.

We conclude from our analysis that both the progression and initiation of DNA replication are inhibited by UV irradiation but that there is a significant amount of synthesis past pyrimidine dimers in the template DNA.

MATERIALS AND METHODS

Cells and virus. The TC7 subclone of CV-1 cells, an established line of African green monkey kidney cells (17), was obtained from P. Tegtmeyer. The cells were grown on plastic in Eagle minimal essential medium (GIBCO Laboratories) supplemented with 5% calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml at 37°C in a 5% CO_2 atmosphere. The cells were infected with SV40 (wild-type strain 830 [21], generously supplied by T. Shenk) at a multiplicity of infection of 1 to 10 (as measured by plaque assay). The virus stocks were prepared at a multiplicity of infection of ~0.005. Crude viral lysates were made by freeze-thaw procedures combined with an adsorption-deadsorption concentration step (4) and were stored at −20°C.

Irradiation conditions. Infected TC7 cells were washed two times with prewarmed (37°C) phosphate-buffered saline (138 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and overlaid with 5 ml of prewarmed phosphate-buffered saline. Uncovered dishes were irradiated with a low-pressure mercury germicidal lamp (GE G414-1) with a maximal output at 254 nm and a fluence rate of 0.676 or 2.24 J/m^2 per s (determined by potassium ferrioxalate actinometry [10]). The effective yield of pyrimidine dimers induced in SV40 DNA in vivo was one dimer per SV40 genome per 19 J/m^2 (see below).

Purified DNA or virions were irradiated in buffer placed on a watch glass and stirred by passing a stream of water-saturated air over the top of the solution. When concentrated DNA samples were used, the fluence was corrected for absorption by the sample according to the method described by Smith and Hanawalt (22).

Determination of pyrimidine dimer yield in SV40 DNA. Accurate determination of pyrimidine dimer yield in our in vivo-irradiated SV40 DNA is essential for the interpretation of our experimental results. Therefore, measurements of dimer yield were made by two independent methods, thin-layer chromatography (TLC) and T4 endonuclease V sensitivity. The TLC assay is most accurate at above 5 dimers per genome, and the T4 endonuclease V assay is most sensitive in the range of 0.5 to 3 dimers per genome.

TLC assay. Samples of DNA, labeled with [^{3}H]deoxyribosylthymine ([^{3}H]dT), were hydrolyzed in formic acid, and then thymine-containing dimers were assayed by chromatography on silica gel G (Macherey-Nagel) TLC plates by the method of Reynolds et al. (16). The pyrimidine dimer yield was calculated, taking into account the TT, CT, and CC dinucleotide frequency of SV40 DNA (1) and the relative rates of formation of TT, CT, and CC dimers (20). To verify that encapsidation of the viral DNA does not change the dimer yield, dimer yields were determined for both purified naked DNA and purified virions in the range of UV fluences from 60 to 280 J/m^2. To prepare purified DNA, SV40 DNA was isolated from Hirt extracts (9) of infected TC7 cells and banded on a CsCl gradient containing ethidium bromide. SV40 virions were purified from crude lysates by banding in CSCl gradients. The data in Table 1 show that dimer yields are almost identical for these two forms of SV40 DNA. When SV40 DNA was irradiated intracellularly (under the conditions described above), the dimer yield was reduced almost twofold (Table 1), probably due to shielding by cellular components.

T4 endonuclease V assay. T4 endonuclease V can be used in a sensitive assay for pyrimidine dimers in supercoiled SV40 DNA (19). In our standard assay, viral DNA was digested with an excess of T4 endonuclease V (purified by the procedure of Seawell et al. [19]) for 15 min at 37°C in 50 mM Tris-hydrochloride (pH 7.5)–5 mM EDTA–50 mM NaCl. Additional enzyme was then added, and incubation was continued for 15 min. Samples were then electrophoresed on agarose gels or sedimented on alkaline sucrose gradients to determine the reduction of form I (supercoiled) molecules compared with identical samples incubated in the absence of enzyme. Control experiments indicated that under these conditions, the endonuclease reaction goes to completion in the first 15 min of incubation. Dimer yields determined by the T4 endonuclease V assay for UV fluences of 10 to 30 J/m^2 are in good agreement with those determined by the TLC method (Table 1).

Labeling and extraction of viral DNA. In experiments where a ^{32}P prelabel was used, the $^{32}P_i$ (ICN Pharmaceuticals, Inc.; carrier free) was added to the growth medium (final concentration, 0.4 μCi/ml) at 24 h after infection. To measure SV40 DNA synthesis, infected cells were labeled with 150 μCi of [^{3}H]dT (50 to 100 Ci/mmol; New England Nuclear Corp. or Amersham Corp.) per ml of medium. Under these labeling conditions, uptake of [^{3}H]dT into SV40 DNA is essentially

TABLE 1. Dimer yields in SV40 DNA

Assay	J/m^2 per pyrimidine dimer per SV40 genome in:		
	SV40 DNA in vitro	Virion DNA in vitro	SV40 DNA in vivo
TLC	11.5, 10.3	11.9	18.7
T4 endonuclease V	9.6, 10.7	—[a]	19.3

[a] Not done.

linear for at least 3 h. Furthermore, we have shown that UV irradiation of infected cells does not change the size or specific activity of the intracellular thymidine triphosphate pool (Barnett, Landaw, and Dixon, manuscript in preparation). Therefore, the amount of [3H]dT incorporated into the SV40 DNA should accurately reflect the amount of DNA synthesized. At the end of the labeling period, the medium was removed, the cells were washed with phosphate-buffered saline, and the viral DNA was extracted by the method of Hirt (9). In some experiments where a density label was required, 5×10^{-5} M bromodeoxyuridine (BUdR), 2×10^{-5} M fluorodeoxyuridine, and 9×10^{-6} M deoxycytidine (24) were added in preconditioned medium. After addition of BUdR, cells and DNA were exposed only to yellow light.

CsCl equilibrium sedimentation. When samples were to be density analyzed, a modified Hirt extraction of the viral DNA was performed in which CsCl is substituted for NaCl. The supernatants were then adjusted to a refractive index of 1.4015 to 1.4025 by addition of solid CsCl and TE buffer (10 mM Tris-hydrochloride, 1 mM EDTA [pH 7.5]) and centrifuged for 60 h at 38,000 rpm and 18°C in a Beckman 50 Ti rotor. Gradients were fractionated by pumping from the bottom of the tube. Radioactivity profiles were determined by trichloroacetic acid precipitation of samples of the gradient fractions.

Form I DNA was isolated by banding DNA from Hirt supernatants in CsCl equilibrium gradients containing 100 μg of ethidium bromide per ml and having a refractive index of 1.3875 to 1.3880.

For banding in alkaline CsCl equilibrium gradients, samples were adjusted to 10 mM NaOH and a refractive index of 1.406 by addition of solid CsCl, 100 mM Na_2HPO_4, 100 mM NaOH, and 2 mM EDTA. The samples were centrifuged in polyallomer tubes at 23°C for 60 h at 38,000 rpm in a 50 Ti rotor.

Alkaline sucrose gradients. Alkaline sucrose gradients (5 to 20%) contained 5 mM EDTA, 0.3 M NaOH, and 0.75 M NaCl. Centrifugation was for 2.5 h at 41,000 rpm and 4°C in a Beckman SW41 rotor.

Agarose gel electrophoresis. For direct analysis of the viral DNA extracted by the Hirt procedure, the supernatants were passed through a small Sephadex G-50 column (Sigma Chemical Co.) equilibrated with TE buffer to reduce the salt concentration (15). The eluate was adjusted to 0.5% sodium dodecyl sulfate and 5% glycerol and immediately applied to a 1% agarose gel. All agarose gels were prepared and electrophoresed with 40 mM Tris–4.7 mM acetate 1 mM EDTA (pH 7.9) for 4 h at 80 mA. Gels were stained with ethidium bromide to visualize DNA bands. Gel lanes were cut into 0.5-cm slices, the slices were solubilized by heating to 110°C in 0.5 ml of 0.1 N HCl for 15 min, and then 5 ml scintillation fluid (RPI 3a70B; Research Products International) was added, and ^3H and ^{32}P radioactivities were determined by scintillation spectrometry.

***Eco*RI digestion.** Samples were digested for 1 h at 37°C in 50 mM NaCl–5 mM $MgCl_2$–100 mM Tris-hydrochloride (pH 7.5) with an excess of *Eco*RI (Bethesda Research Laboratories). Digestion was terminated by addition of EDTA to 20 mM.

BND-cellulose chromatography. The procedure of Tapper and DePamphilis (23) was followed for benzoylated napthoylated DEAE-cellulose (BND-cellulose) chromatography. CsCl-purified SV40 DNA (in TE buffer plus 0.3 M NaCl) from two 100-mm dishes of infected cells was applied to a 0.5-ml BND-cellulose column which had been previously washed with 20 ml of TE buffer plus 0.3 M NaCl. The columns were then eluted stepwise with 20 ml of TE buffer plus 0.65 M NaCl followed by 20 ml of TE buffer plus 1.0 M NaCl plus 2% caffeine. Columns were loaded and developed at 37°C in order for the caffeine to remain in solution at this concentration. The flow rate was approximately 1 ml/min. Greater than 50% of the radioactivity eluting in each fraction was present in the first 4 ml of the 20-ml eluate.

RESULTS

Rate of SV40 DNA replication after UV irradiation. To determine the immediate short-term effects of low-fluence UV radiation on the rate of SV40 DNA replication, we measured the incorporation of [^3H]dT into viral DNA in 15-min pulses during the first 45 min after irradiation (0 to 60 J/m^2) of infected cells. In these experiments irradiation was carried out 36 h after infection since the rate of SV40 DNA replication is maximal at this time under our conditions. To determine the amount of label incorporated specifically into viral DNA, cellular DNA was precipitated by the method of Hirt (9), and supernatants containing primarily viral DNA were electrophoresed on agarose gels (Fig. 1B). The results in Fig. 1 indicate that there is a fluence-dependent and time-dependent reduction in viral DNA synthesis after UV irradiation of infected cells. Furthermore, the completion of SV40 replication to produce form I molecules is more severely inhibited than is overall replication. The results in Table 2 show that the rate of replication of SV40 DNA continues to decline for up to 3 h after UV irradiation of infected cells. Furthermore, completion of form I molecules continues to be more severely inhibited than total DNA synthesis.

Effect of UV irradiation on replication pools. To obtain a more detailed picture of the way in which SV40 DNA replication is inhibited by UV radiation, we examined further the distribution of replicative intermediates after irradiation of infected cells. Infected cells were prelabeled with $^{32}P_i$ to provide an internal control for recovery, and then at 36 h after infection, cells were irradiated and labeled for 15, 30, or 45 min with [^3H]dT. Viral DNA was isolated by Hirt extraction followed by CsCl equilibrium sedimentation and then fractionated by BND-cellulose chromatography. Form I is eluted first from BND-cellulose with 0.65 M NaCl, and then molecules with single-stranded regions (e.g., replicative intermediates) are eluted with 2% caffeine–1.0 M NaCl (23). The amount of ^3H label in the different forms was determined and then normalized for recovery by using the ^{32}P-labeled standard.

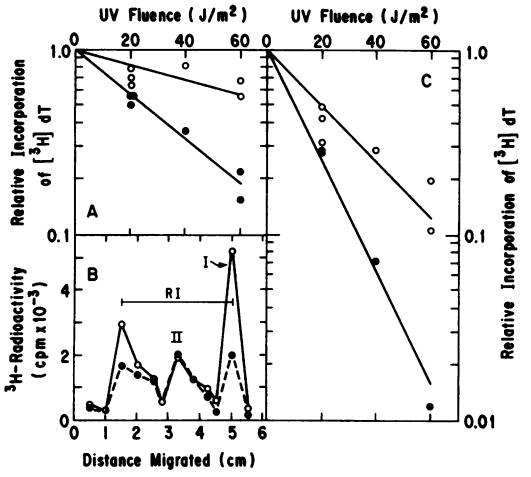

FIG. 1. Incorporation of [^{3}H]dT into SV40 DNA after UV irradiation of infected cells. SV40-infected cells were labeled with $^{32}P_i$ beginning at 24 h after infection. At 36 h after infection, cells were UV irradiated (UV fluence rate, 0.676 J/m^2 per s) and then pulse-labeled for 15 min beginning either immediately after UV irradiation (A) or 30 min after UV irradiation (C). Labeled cells were extracted by the method of Hirt (9) and electrophoresed directly on agarose gels as described in the text. Typical gel profiles are displayed in panel B (○, unirradiated control labeled from 30 to 45 min after mock UV irradiation; ●, cells irradiated at 20 J/m^2 and labeled from 30 to 45 min after UV irradiation). Total ^{3}H radioactivity is normalized to ^{32}P radioactivity to adjust for recovery. Unirradiated samples contained 5×10^5 cpm of ^{3}H per dish on the average. Note that positions of the gel slices are chosen on the basis of ethidium bromide staining, and not all slices are the same size. Total ^{3}H radioactivity (open circles in panels A and C) in SV40 DNA was determined by summing the ^{3}H radioactivity over the gel from the top through the SV40 form I band. Radioactivity in SV40 form I (closed circles in panels A and C) was taken as the ^{3}H radioactivity in the single form I gel slice (see panel B). Incorporation of ^{3}H radioactivity in UV-irradiated samples is plotted relative to that in unirradiated controls. Data were compiled from three separate experiments.

Again (Fig. 2A) it can be seen that the label incorporated into form I decreases as a function of UV fluence. However, label incorporation into replicative intermediates is much less affected by UV radiation (Fig. 2B). Since both the ^{32}P label and the ^{3}H label in replicative intermediates are relatively unaffected by UV radiation, we conclude that the size of the replicative-intermediate pool remains relatively constant after UV irradiation.

Pyrimidine dimer content of newly replicated molecules. To determine whether pyrimidine dimers are complete blocks to viral DNA replication, we asked whether dimer-containing molecules are ever completely replicated after UV irradiation. To answer this question it was first necessary to examine specifically only those molecules that were initiated and completely replicated after UV irradiation. This requires that such molecules be separated from the bulk of nonreplicated DNA and also from molecules that were in the process of replication at the time of irradiation and were completed thereafter. These latter molecules could contain dimers in segments that had already replicated at the time of UV irradiation. Once newly replicated molecules were isolated, their dimer content could be measured.

Two different experimental protocols were used. In experiment 1, BUdR was added immediately after irradiation of infected cells to permit density labeling of newly replicated molecules, and [^{3}H]dT was added 30 min thereafter. After an additional 2.5 h, viral DNA was extracted and sedimented on CsCl equilibrium gradients (Fig. 3A). Thus, molecules completely replicated after UV irradiation should appear as ^{3}H-labeled molecules banding at a fully hybrid (HL) density. Both the ^{3}H label and the BUdR label should be contained in the newly synthesized daughter strands. Since a 30-min time period (sufficient for two rounds of replication under normal conditions) was allowed between irradiation and addition of the ^{3}H label, few molecules whose replication was initiated before irradiation should be labeled. Furthermore, since the HL and light-density (LL) peaks are separated by about 10 fractions, material contained in the HL fraction should be almost exclusively HL molecules.

In experiment 2, infected cells were pulse-labeled with [^{3}H]dT for 1 h before irradiation, and BUdR was added 30 min after irradiation. Again, viral DNA was extracted 2.5 h later and

TABLE 2. Incorporation of [^{3}H]dT into SV40 DNA after UV irradiation (20 J/m^2) of infected cells

Type of labeling	End of labeling period (min)	[^{3}H]dT incorporation[a] in:	
		Total DNA	Form I DNA
15-min pulse	15	0.60	0.41
	45	0.16	0.05
	120	0.06	<0.01
	180	0.01	<0.01
Continuous[b]	30	1.06	0.60
	60	0.64	0.46
	120	0.39	0.33

[a] Relative to unirradiated controls. Values have been normalized for recovery by using a ^{32}P prelabel.
[b] The [^{3}H]dT label was added in the presence of BUdR, fluorodeoxyuridine, and deoxycytidine since samples were being prepared for density separations as well.

sedimented on CsCl equilibrium gradients (Fig. 3B). The HL peak was then rebanded on a CsCl gradient (Fig. 3C) to remove any residual contamination of LL DNA. In this case, the radioactive label should be present in dimer-containing parental strands, whereas the density label should be present in newly synthesized daughter strands.

In both experiments, conversion of dimer-containing HL molecules from form I to form II (relaxed circles) by T4 endonuclease V treatment was monitored by alkaline sucrose gradient sedimentation (examples are shown in Fig. 4). The results of these experiments (Table 3) clearly demonstrate that a large proportion of DNA molecules replicated after UV irradiation contain pyrimidine dimers. In experiment 1, where infected cells received either 20 or 40 J/m^2 (about one or two dimers per SV40 genome), the proportion of newly replicated molecules that contained dimers at 3 h after irradiation was 0.38 to 0.39 and 0.63, respectively. The number of replicated dimer-containing molecules apparently increases as a function of time after UV irradiation, since only 0.19 of HL form I molecules isolated at 30 min after irradiation were sensitive to T4 endonuclease V. Control samples which were treated identically except that UV irradiation was omitted exhibited very little (only 5%) conversion of form I to form II after T4 endonuclease V treatment, indicating that neither the presence of BUdR in the DNA nor the presence of contaminating nucleases can account for the T4 endonuclease V sensitivity of the DNA from UV-irradiated cells.

In experiment 2, about 0.42 of the HL molecules present at 3 h after UV irradiation (20 J/m^2) were sensitive to T4 endonuclease V. In this experiment, DNA that did not replicate after UV irradiation could be analyzed as well to determine its dimer content both immediately after UV irradiation and 3 h later. In both cases it was found to have the expected proportion of dimer-containing molecules (about 0.62) for the UV fluences used. Thus, there appears to be little or no excision repair of these molecules during the time course of the experiment.

Entry of molecules into the replication pool. In addition to confirming that dimer-containing molecules can become fully replicated, experiment 2 also provided information on the extent to which entry of new molecules into the replication pool is inhibited by UV irradiation. In the initial CsCl banding of DNA from unirradiated cells (Fig. 3B), about 75% of the ^{3}H-labeled DNA banded at the LL density; the remainder (25%) banded at the HL density. This indicates that about 25% of previously replicated molecules (^{3}H labeled) reenter and complete replication during the BUdR labeling period (30 min to 3 h after mock irradiation). In contrast, in samples from UV-irradiated cells, over 90% of the ^{3}H label remained at the LL density, and only about 4% banded at the HL density. The fact that considerably more DNA appears to remain at the LL density position in the UV-irradiated samples indicates that not only is completion of damaged DNA slowed, but the entry of DNA into the replication pool appears to be slowed as well.

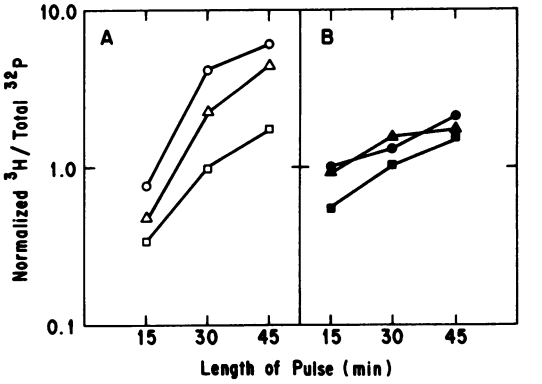

FIG. 2. Incorporation of [^{3}H]dT into SV40 completed molecules and replicative intermediates after UV irradiation of infected cells. BND-cellulose chromatography was performed as described in the text. Total ^{3}H radioactivity in the eluate of TE buffer plus 0.65 M NaCl (A) or of TE buffer plus 1.0 M NaCl plus 2% caffeine (B) was determined. This value was divided by the total ^{32}P radioactivity present in the sample (i.e., ^{32}P in A plus B for a given UV dose and pulse duration) to correct for recovery of total SV40 DNA. These ^{3}H/^{32}P ratios were further normalized to a value of 1.0 for the eluate of TE buffer plus 1.0 M NaCl plus 2% caffeine for SV40 DNA from a 15-min pulse in unirradiated cells to allow averaging of data from multiple repetitions of the experiment. Values plotted are averages of three determinations for zero dimers per genome, two determinations for one dimer per genome, and a single determination for two dimers per genome. Determinations varied among different experiments by an average of 35% for form I and 14% for replicative intermediates, but this variation did not alter significantly the shapes of the curves. Total recovery of ^{3}H-labeled material from the BND-cellulose columns was always between 75 and 80%. UV doses (J/m^2) were 0 (○,●), 20 (△,▲), and 40 (□,■).

Recombination control. To confirm that the HL molecules containing dimers actually arose by replication on damaged templates, it is necessary to rule out the possibility that they result from recombination between unreplicated, damaged templates and replicated undamaged templates. This appears to be particularly important because it has been shown that UV irradiation of SV40 enhances genetic recombination 25- to 40-fold (5). To test for the presence of recombinant

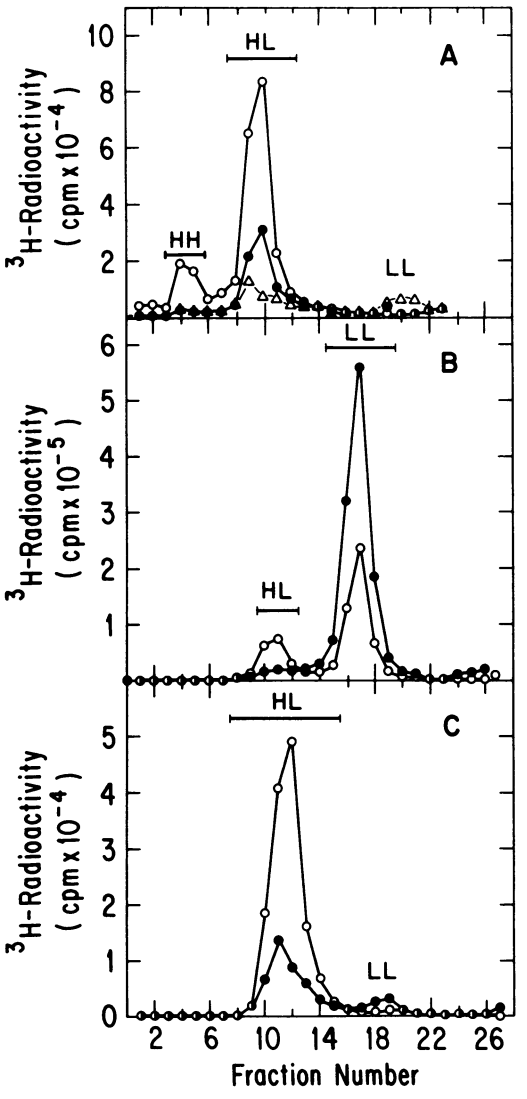

FIG. 3. Equilibrium sedimentation of newly synthesized HL viral DNA on neutral CsCl gradients. SV40 DNA was extracted from infected cells and sedimented in neutral CsCl equilibrium density gradients as described in the text. Radioactivity in gradient fractions was determined by trichloroacetic acid precipitation of DNA and scintillation counting. Fractions pooled for pyrimidine dimer determinations are indicated by the horizontal bars. HH, HL, and LL indicate the banding positions of heavy-density, hybrid-density, and light-density DNA, respectively. (A) Experiment 1. BUdR was added immediately after UV irradiation (24 h after infection), [^{3}H]dT was added 30 min thereafter, and samples were prepared 2.5 h later. Gradients contained DNA extracted from two, four, or three dishes for samples irradiated with 0, 20, or 40 J/m^2, respectively. (B) Experiment 2. Infected cells were pulse-labeled (24 h after infection) with [^{3}H]dT for 1 h before UV irradiation, BUdR was added at 30 min after UV irradiation, and samples were prepared 2.5 h later. Gradients contained DNA extracted from two or six dishes for samples irradiated with 0 or 20 J/m^2, respectively. (C) Rebanding of HL fraction shown in panel B. Symbols: ○, DNA from unirradiated cells; ●, DNA from cells UV irradiated at 20 J/m^2; △, DNA from cells UV irradiated at 40 J/m^2.

molecules under our experimental conditions, HL DNA molecules were isolated as described above for experiment 1, linearized with *Eco*RI, denatured, and banded on alkaline CsCl gradients. Under these conditions, the parental strands should be unlabeled and band at light (L) density, and the newly synthesized daughter strands should be ^{3}H labeled and band at heavy density. The DNA strands of recombinants should be radioactively labeled and band at intermediate density. The alkaline CsCl gradient profiles are shown in Fig. 5. The separated strands of HL DNA from UV-irradiated or unirradiated cells appear to be homogeneous in density and band at the same position as separated strands from fully heavy DNA. (The very slight differences in banding positions of the DNAs is not significant since, in a repeat of this experiment, the position of the DNA from irradiated cells was shifted very slightly toward the heavier density.) Thus, the HL molecules selected for dimer content determination do not contain a significant number of recombinant, intermediate-density strands. Therefore, we conclude that the presence of dimers in a large proportion of this DNA cannot be explained by either an enhanced molecular exchange caused by UV irradiation or an insertion of large segments of fully LL DNA into replicated molecules.

This conclusion was confirmed in another experiment (data not shown) where the HL DNA fraction (prepared as for Fig. 3A) was cleaved with T4 endonuclease V and then sedimented on an alkaline sucrose gradient. The ^{3}H-labeled DNA that sedimented as single strands (presumably single-stranded circles) was then banded on alkaline CsCl gradients. This ^{3}H-labeled DNA migrated at the same position as unirradiated control DNA cleaved with *Eco*RI.

DISCUSSION

Our experimental results demonstrate that exposure of SV40-infected cells to low fluences of UV radiation leads to inhibition of SV40 DNA replication. The rate of production of newly completed form I molecules decreases dramatically as a function of both time after UV irradiation and UV fluence to levels below those expected for continued replication of undamaged templates. Although some dimer-containing molecules appear to be completed within a short time after UV irradiation, many molecules remain in the pool of replicative intermediates longer

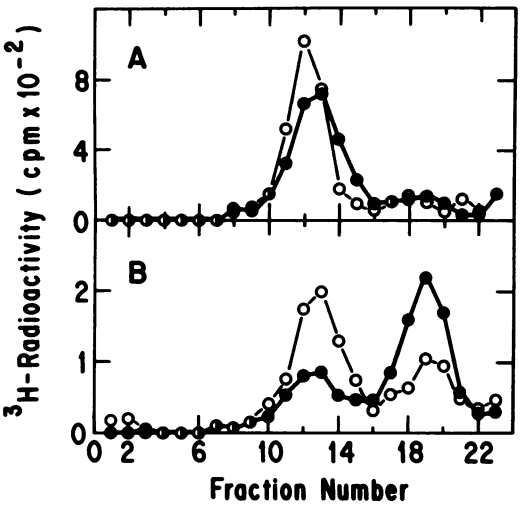

FIG 4. Alkaline sucrose velocity sedimentation of T4 endonuclease V-digested newly synthesized HL viral DNA. The HL fractions (see Fig. 3B) for DNA from unirradiated (A) or UV-irradiated (20 J/m^2; B) cells were digested with T4 endonuclease V. Samples were layered onto alkaline sucrose gradients as described in the text. Samples were incubated in absence of enzyme (○) or in the presence of T4 endonuclease V (●). The proportion of form I remaining (peak centered around fractions 12 to 13) after digestion is reported in Table 3, experiment 2, HL DNA.

TABLE 3. Pyrimidine dimers in replicated molecules

Expt	Time (h) of labeling with: BUdR	Time (h) of labeling with: [^{3}H]dT	UV fluence (J/m^2)	DNA density	Fraction with ≥1 dimer[a]
1	0–3	0.5–3	20	HL	0.38, 0.39[b]
	0–3	0.5–3	40	HL	0.63
	0–0.5	0–0.5	20	HL	0.19
2	0.5–3	−1–0	20	HL	0.42
	0.5–3	−1–0	20	LL	0.62
	0 (^{32}P prelabel)	0 (^{32}P prelabel)	20	LL	0.62

[a] Fraction of form I converted to form II by treatment with T4 endonuclease V, minus the fraction converted in unirradiated controls. The control values were 0.05 and 0.08 for experiments 1 and 2, respectively. Digestion was with 50 μl of enzyme and 6 ng of DNA in a total volume of 100 μl. Approximately 70% of the total DNA was form I before digestion.

[b] HL DNA was rebanded on a CsCl density gradient containing ethidium bromide to purify form I DNA before T4 endonuclease V treatment.

than normal. Dimer-containing molecules continue to be completed at a slow rate after UV irradiation, but replication of some damaged molecules may be permanently blocked. Entry of new molecules into the pool is reduced, perhaps due to a coupling between completion of old molecules and initiation of new ones. The accumulation of blocked molecules and the failure of new molecules to enter the replication pool would account for the observed progressive reduction in overall SV40 DNA replication.

Inhibition of DNA replication. Since there is a progressive decline in replication rate with increasing time after UV irradiation, the apparent inhibition observed depends on the experimental conditions used. Taking these differences in experimental conditions into account, our results on the inhibition of DNA replication by UV radiation are in general agreement with those of Williams and Cleaver (25) and Sarasin and Hanawalt (18), who carried out similar investigations.

Inhibition of initiation. We have demonstrated that entry of new molecules into the replication pool is inhibited after UV irradiation. Since the size of the replication pool appears to remain relatively constant, we postulate that the inhibition of entry is due to the failure to complete molecules already in the pool and that initiation of replication of new molecules is in some way tied to completion of others. The simplest explanation for this would be that some factor(s) required for initiation is present in limiting amounts and remains associated with blocked molecules. It is interesting to note that initiation of cellular replicons also appears to be inhibited after UV irradiation of uninfected cells, as indi-

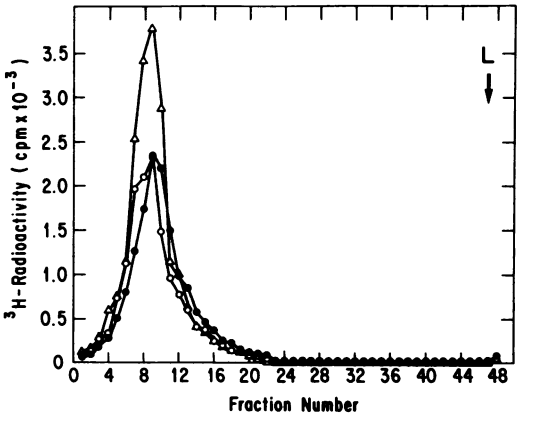

FIG 5. Sedimentation on alkaline CsCl equilibrium gradients of HL SV40 DNA. The HL fractions for DNA from UV-irradiated (●; 20 J/m^2) or unirradiated (○) cells and heavy-density fractions from unirradiated cells (△) from neutral CsCl gradients similar to the ones shown in Fig. 3A were dialyzed, digested with *Eco*RI, and banded in alkaline CsCl equilibrium gradients. The fractions were neutralized, and the radioactivity was determined.

cated by the inhibition of incorporation of [^{3}H]dT into DNA strands of less than full replicon size (11). Our conclusion on inhibition of initiation differs from that of Williams and Cleaver (25). They conclude that "initiation was not photosensitive because the relative proportion of RI [replicative-intermediate] molecules did not decrease with increasing UV dose." However, they did not measure initiation of new molecules in any direct way.

Dimers in newly replicated molecules. We have demonstrated that a large proportion of molecules completed after UV irradiation contain pyrimidine dimers. Sarasin and Hanawalt made a similar observation using different experimental conditions (18). They used a *tsA* mutant and temperature shifts so that presumably no molecules were in the process of replication at the time of UV irradiation. Our confirmation of this result under more normal growth conditions eliminates the possibility that their observations on the replication of dimer-containing molecules were due to perturbations of the replication machinery induced by incubations of cells at high or low temperature. Williams and Cleaver (25) also concluded that dimer-containing molecules must be replicated, since the majority of a 20-min pulse-label could be chased into completed molecules. However, they made no direct measurements of the dimer content of newly replicated molecules.

Recombination. Recombination of genetic markers is enhanced by UV irradiation of SV40 (5). Thus, it was necessary to consider the possibility that the dimers present in newly replicated molecules were actually derived by recombination from dimer-containing unreplicated molecules. In the simplest case this would involve homologous recombinational events in which sizable segments of double-stranded DNA would be exchanged between newly replicated and unreplicated molecules. This type of event is known to occur at high frequency in bacterial systems (6). Our experimental results demonstrate that this type of event does not occur at high frequency after UV irradiation of SV40-infected cells. We calculate that insertion of 275 base pairs of fully LL DNA should have caused a shift in the density of a heavy strand so that it would have sedimented two fractions lighter in Fig. 5. Therefore, it appears that the majority of dimers present in newly replicated molecules cannot be accounted for by recombinational insertions of unreplicated DNA segments longer than 275 base pairs. Sarasin and Hanawalt assayed newly synthesized daughter strands for the presence of dimers by using T4 endonuclease V and strand size analysis on sucrose gradients. Although this assay was not as sensitive as ours, they also failed to detect appreciable recombinational insertion of dimers into daughter strands.

It is important to note that these experiments do not bear on the question of whether a postreplicational repair mechanism exists in this system that allows completion of newly replicated daughter strands by the recombinational insertion of small single-stranded DNA fragments opposite damaged sites in the SV40 DNA. Such a mechanism is quite active in bacterial systems (6).

The mechanism by which dimer-containing molecules become completed remains unclear. Several different models have been proposed (7, 8, 12), but presently there is little concrete evidence to support or refute any of them. Further analysis of the molecular structure of blocked replicative intermediates that accumulate after UV irradiation and analysis of the position and strand specificity of dimers in completed molecules should provide a basis for proposing specific models for dimer accommodation. Present work in our laboratory is aimed at determining the mechanism by which dimers are accommodated after UV irradiation of SV40-infected cells.

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